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The C-terminal tail of *Arabidopsis* 14-3-3ω functions as an autoinhibitor and may contain a tenth α-helix

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Summary

The eukaryotic regulatory protein 14-3-3 is involved in many important plant cellular processes including regulation of nitrate assimilation through inhibition of phosphorylated nitrate reductase (pNR) in darkened leaves. Divalent metal cations (Me^{2+}) and some polyamines interact with the loop 8 region of the 14-3-3 proteins and allow them to bind and inhibit pNR *in vitro*. The role of the highly variant C-terminal regions of the 14-3-3 isoforms in regulation by polycations is not clear. In this study, we carried out structural analyses on the C-terminal tail of the *Arabidopsis* 14-3-3ω isoform and evaluated its contributions to the inhibition of pNR. Nested C-terminal truncations of the recombinant 14-3-3ω protein revealed that the removal of the C-terminal tail renders the protein partially Mg^{2+}-independent in both pNR binding and inhibition of activity, suggesting that the C-terminus functions as an autoinhibitor. The C-terminus of 14-3-3ω appears to undergo a conformational change in the presence of polycations as demonstrated by its increased trypsin cleavage at Lys-247. C-terminal truncation of 14-3-3ω at Thr-255 increased its interaction with antibodies to the C-terminus of 14-3-3ω in non-denaturing conditions, but not in denaturing conditions, suggesting that the C-terminal tail contains ordered structures that might be disrupted by the truncation. Circular dichroism (CD) analysis of a C-terminal peptide, from Trp-234 to Lys-249, revealed that the C-terminal tail might contain a tenth α-helix, in agreement with the *in silico* predictions. The function of the putative tenth α-helix is not clear because substituting two prolyl residues within the predicted helix (E245P/I246P mutant), which prevented the corresponding peptide from adopting a helical conformation, did not affect the inhibition of pNR activity in the presence or absence of Mg^{2+}. We propose that in the absence of polycations, access of target proteins to their binding groove in the 14-3-3 protein is restricted by the C-terminus, which acts as part of a gate that opens with the binding of polycations to loop 8.

Keywords: 14-3-3 proteins, nitrate reductase, autoinhibition, protein structure, protein–protein interaction, protein phosphorylation.

Introduction

The eukaryotic 14-3-3 proteins are emerging as an important class of regulatory proteins that are involved in many aspects of cellular processes (for recent reviews see Roberts, 2000; Sehnke *et al.*, 2002; Tzivion and Avruch, 2002; Yaffe, 2002). In most cases, the 14-3-3 proteins execute their functions by recognizing and binding to specific phospho-Ser- or phospho-Thr-containing motifs, RXX(pS/pT)XP or RXXX(pS/pT)XP, in their target proteins, providing an important link between the post-translational regulation by protein phosphorylation and its downstream effects (Sehnke *et al.*, 2002). However, the 14-3-3s have other recognition sequences that may not contain a phosphorylated amino acid residue (Tzivion and Avruch, 2002; Yaffe, 2002). Upon binding to their target proteins, the 14-3-3s can alter the activities of a number of enzymes, many of which are involved in metabolism in plants (for reviews see Chung *et al.*, 1999; Roberts, 2000), including nitrate reductase (Bachmann *et al.*, 1996a,b; Moorhead *et al.*, 1996), sucrose-phosphate synthase (SPS; Moorhead *et al.*, 1999; Toroser *et al.*, 1998), and plasma membrane H^+-ATPase (Korthout and de Boer, 1994; Marra *et al.*, 1994; Oecking *et al.*, 1994). Some 14-3-3 proteins are also implicated in controlling gene expression as they bind to the transcription factors such as EmBP and VP1 in the nucleus (Schultz *et al.*, 1998) or to other proteins functioning in

transcription initiation (Lu *et al.*, 1992; Pan *et al.*, 1999). On the other hand, by binding to the transcriptional activator RGS, which is involved in gibberellin biosynthesis, a tobacco 14-3-3 isoform can sequester it in the cytosol and prevent it from entering the nucleus to regulate the gene expression (Igarashi *et al.*, 2001). Because 14-3-3 proteins exist as homo- or heterodimers and both subunits can bind a target protein, they sometimes function as molecular scaffolds to bring two proteins together, as demonstrated by the interaction of the animal protein kinase Raf and its partners that resulted from their binding to a single 14-3-3 dimer (for reviews see Tzivion and Avruch, 2002; Yaffe, 2002). All these observations indicate a pivotal role of 14-3-3 proteins in signal transduction.

In higher eukaryotes, 14-3-3 proteins are encoded by small gene families. Fifteen 14-3-3 genes have been identified in the *Arabidopsis* genome, and at least 12 of these genes are expressed, as their cDNA clones have been obtained (Rosenquist *et al.*, 2001). The 14-3-3s are acidic proteins with a monomer size of around 30 kDa. The overall amino acid sequences of the 14-3-3 proteins are overwhelmingly conserved not only among the isoforms from a single species, but also between the different kingdoms (Rosenquist *et al.*, 2000, 2001). This may reflect the conservation in the mechanism of the 14-3-3 action as well as the fundamental importance of the 14-3-3 functions. Thus, the crystal structures determined for the human 14-3-3 τ and ζ isoforms (Liu *et al.*, 1995; Obsil *et al.*, 2001; Xiao *et al.*, 1995) can be extended to the plant 14-3-3 isoforms. These three-dimensional structures reveal that the 14-3-3 proteins contain nine α-helices throughout the conserved region, and that the two monomers interact through their N-termini to form a clamp-like structure with two inner surfaces for target protein binding. However, the structure of the 14-3-3 C-terminal tail, which is an exceptional region with substantial sequence divergence and size variation among the isoforms, remains elusive. The apparent lack of conservation in this part of the 14-3-3 protein compared with the high degree of conservation in the rest of the protein is intriguing (Chung *et al.*, 1999). Divergence in the C-terminal tails among 14-3-3 isoforms suggests the possibility that it is a part of the structural basis for determining certain specific functions of the 14-3-3 proteins, for instance, the specificity of interaction between a 14-3-3 protein and its target proteins. Some of the *Arabidopsis* 14-3-3 isoforms such as λ, κ, and have very short C-terminal tails, and it seems that this region is dispensable for 14-3-3 function. However, the importance of the C-terminal tail is suggested by the observation that its complete removal prevents the inhibition of phosphorylated nitrate reductase (pNR) by *Arabidopsis* 14-3-3ω (Athwal and Huber, 2002). Another possibility is that the C-terminal tail may undergo post-translational processing, for example, truncation (Testerink *et al.*, 2002; van Zeijl *et al.*, 2000), which can alter its activity in target protein regulation. Moreover, it is an open possibility that this region can also mediate specific interactions with other cellular factors that will control the activity of the 14-3-3 proteins.

We have been using pNR as a model system for studying how the 14-3-3 proteins are regulated in interaction with their target proteins. NADH:nitrate reductase (NR) catalyzes the first step in nitrate assimilation and its activity should be tightly controlled because its product, nitrite, is toxic to the plant cells, and can accumulate in leaves in conditions such as darkness when nitrite reductase, the enzyme that converts nitrite to ammonia, is also downregulated (for reviews see Huber *et al.*, 1996; Kaiser and Huber, 2001; Kaiser *et al.*, 2002; MacKintosh and Meek, 2001). Therefore, the rapid control of NR activity is essential. In addition to the transcriptional regulation of the NR genes by light and nitrate availability, the NR protein can be rapidly and reversibly phosphorylated at the Ser-543 site in its hinge 1 region and subsequently inactivated by binding to the 14-3-3 proteins (Bachmann *et al.*, 1996c; Moorhead *et al.*, 1996). Previous investigations showed that at pH 7.5, divalent metal cations (Me^{2+}) or certain polyamines are required for 14-3-3 proteins to inhibit pNR, but at low pH, for example pH 6.5, such 14-3-3-mediated inhibition of pNR is largely polycation-independent (Athwal *et al.*, 1998a,b, 2000; Bachmann *et al.*, 1996b). The reduced dependence on Me^{2+} at pH 6.5 reflects the 'H^+-activation' of the 14-3-3 binding to the targets (Athwal *et al.*, 2000). The loop 8 region of the 14-3-3 protein has been implicated in mediating Me^{2+}-binding (Athwal and Huber, 2002; Lu *et al.*, 1994), but the basis for H^+-activation is not clear. In the present study, we focused on the variant C-terminal tail region of the 14-3-3 protein. Using the *Arabidopsis* 14-3-3ω isoform, we show that the removal of its C-terminal tail renders the protein partially Me^{2+}-independent in both pNR binding and inhibition, suggesting that the C-terminal tail functions as an autoinhibitor in agreement with the observations recently reported for the human 14-3-3ζ isoform (Truong *et al.*, 2002). We further demonstrate that the C-terminal tail of the 14-3-3ω isoform might contain a tenth α-helix, which was not resolved in the crystal structures of the human 14-3-3 isoforms. We also show that the structural basis for the activation of 14-3-3ω by polycation binding includes a conformational change in the C-terminus as demonstrated by its increased trypsin cleavage at Lys-247 in the presence of spermine as well as Mg^{2+} or Ca^{2+}. A 'gate-opening' model is proposed for the role of the C-terminal tail of 14-3-3ω in its activation by polycation binding at the loop 8 region.

Results

The C-terminal tail of Arabidopsis *14-3-3ω is an autoinhibitor*

The importance of the non-conserved C-terminal tail region of the 14-3-3 proteins in their action on target proteins has

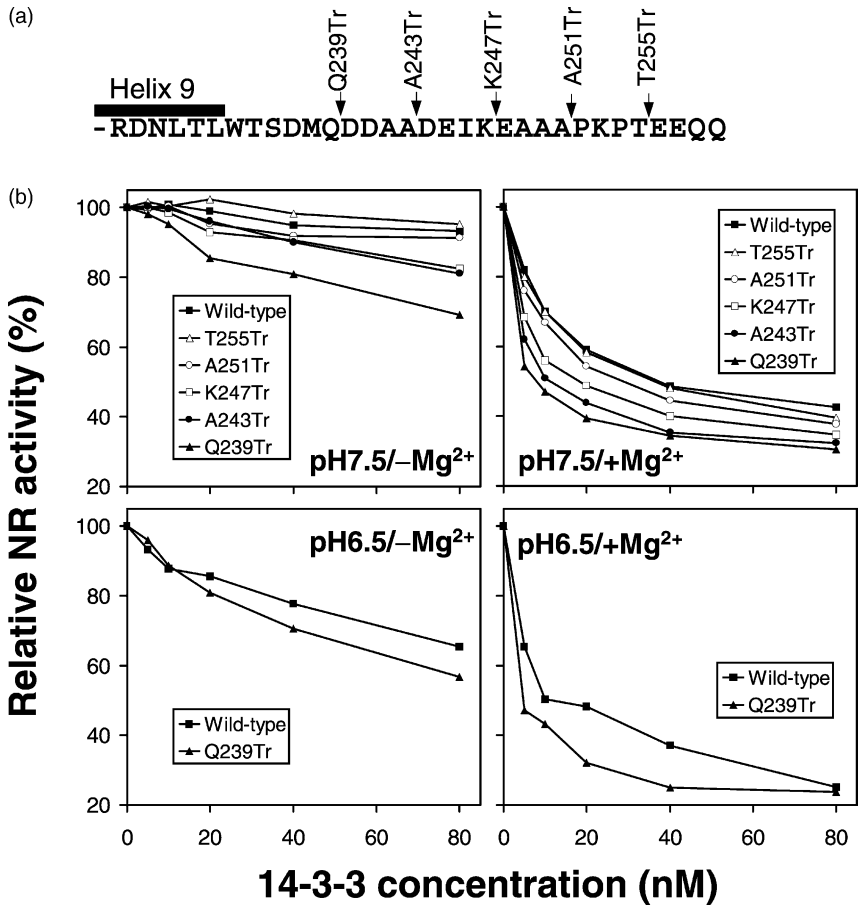

Figure 1. Inhibition of pNR activity by C-terminal truncations of *Arabidopsis* 14-3-3ω.
(a) The positions of the 14-3-3ω C-terminal truncations, T255Tr, A251Tr, K247Tr, A243Tr, and Q239Tr, are shown.
(b) Inhibition of pNR from spinach leaves by increasing concentrations (up to 80 nM) of the dimeric wild-type and C-terminal truncated forms of 14-3-3ω. The assay conditions were: pH 7.5 without $MgCl_2$ but with 1 mM EDTA (pH 7.5/–Mg^{2+}); pH 7.5 with 5 mM $MgCl_2$ (pH 7.5/+Mg^{2+}); pH 6.5 without $MgCl_2$ but with 1 mM EDTA (pH 6.5/–Mg^{2+}); or pH 6.5 with 5 mM $MgCl_2$ (pH 6.5/+Mg^{2+}).
Data are expressed as percentage of pNR activity in the absence of 14-3-3 proteins. Results are representative of at least three separate experiments.

not been clearly determined. Athwal and Huber (2002) showed that the C-terminal truncation of *Arabidopsis* 14-3-3ω at Trp-234, which is at the boundary of helix 9 and the C-terminal tail, rendered it completely inactive in pNR inhibition. An earlier report demonstrated that the removal of up to five residues from the C-terminal end of human 14-3-3τ did not affect its binding to the target proteins, but the deletion of 15 residues from the end greatly reduced the target protein binding (Liu *et al.*, 1996). In contrast, removal of the C-terminal 15 amino acid residues from human 14-3-3ζ was recently shown to enhance the binding to its target proteins (Truong *et al.*, 2002). To precisely determine the minimal extent of the C-terminus that is required for 14-3-3ω to inhibit pNR, we produced recombinant 14-3-3ω proteins with nested C-terminal truncations. Each of the truncated forms, T255Tr, A251Tr, K247Tr, A243Tr, and Q239Tr, had four amino acid residues removed consecutively from the C-terminal end of the wild-type 14-3-3ω protein (Figure 1a). The K247Tr, A243Tr, and Q239Tr forms were able to inhibit pNR in the absence of Mg^{2+} at pH 7.5, with Q239Tr being the most effective, reaching 30% inhibition at 80 nM (Figure 1b). Thus, the C-terminal tail of 14-3-3ω from Asp-240 to Gln-259 is not only dispensable for pNR inhibition, but also functions as an autoinhibitor, preventing interaction with pNR in the absence of cation effectors.

The autoinhibitory function of the C-terminal tail was more obvious when 5 mM $MgCl_2$ was included in the NR activity assay. All the truncated forms of 14-3-3ω except T255Tr were more inhibitory than the wild-type protein, and the increase of the pNR inhibition activity correlated with the extent of truncation (Figure 1b). The effect of the C-terminal truncation on the elevation of pNR inhibition activity was already pronounced at 14-3-3 concentrations of 5 nM. Although the truncated forms of 14-3-3ω showed Mg^{2+}-independent inhibition on pNR, the truncations did not eliminate the interaction of Mg^{2+} with the 14-3-3 protein; for example, Q239Tr was more inhibitory in the presence of Mg^{2+} than in the absence of Mg^{2+} (Figure 1b, compare the top two charts). The Mg^{2+} concentration required for reaching 50% of the maximal pNR inhibition was 4.8 and 3.7 mM for the wild-type and the Q239Tr truncation, respectively (data not shown), indicating that the Mg^{2+}-binding affinity was not altered by the truncation. Moreover, the truncation at Gln-239, but not at Trp-234 (Athwal and Huber, 2002), retained the ability to inhibit the pNR activity in the presence of Mg^{2+}, which suggested that the region between Thr-235 and Gln-239 was critical for 14-3-3 activity (Figure 1b).

Finally, at pH 6.5, the Q239Tr truncated form of 14-3-3ω also showed increased pNR inhibition activity compared

with the wild type, both in the absence and presence of Mg^{2+} (Figure 1b, lower panels). Therefore, the enhancement of 14-3-3-dependent inhibition of pNR by the removal of the C-terminal tail of 14-3-3ω is apparently additive to the H^{+}-activation of the 14-3-3 binding.

The C-terminal tail of 14-3-3ω inhibits binding to pNR in the absence of cations

In Figure 1, the effect of cations on the 14-3-3 function was monitored using the inhibition of pNR activity as the assay. In order to determine whether the C-terminus of the 14-3-3 affects binding to the pNR or influences the pNR activity in some other way, the following experiment was conducted. The $(His)_6$-tagged wild-type 14-3-3ω or Q239Tr C-terminal truncation mutant was immobilized on Ni-iminodiacetic acid (Ni-IDA) resin, and the binding of partially purified pNR was measured. As shown in Figure 2, the binding of pNR to the wild-type 14-3-3ω was stimulated more than fivefold by Mg^{2+}. In contrast, the binding of pNR to the Q239Tr mutant protein was substantial in the absence of Mg^{2+} and was increased slightly by the presence of Mg^{2+}. Thus, the C-terminally truncated form of 14-3-3ω not only had Mg^{2+}-independent pNR inhibition activity, but also showed Mg^{2+}-independent pNR binding. The correspondence between the inhibition of pNR activity and the binding of the 14-3-3 protein suggested that the 14-3-3:pNR complex was inactive, and that the role of Me^{2+} was to promote binding of the 14-3-3 to the pNR.

Polycations alter the accessibility of the 14-3-3ω C-terminus

Although the activation of 14-3-3ω by polycation binding or low pH is independent of the activation by the C-terminal truncation (Figure 1b), the structural basis for activation by cations may still include conformational changes in the C-terminal tail. Lu *et al.* (1994) reported that millimolar concentrations of Ca^{2+} increased the trypsin cleavage at Lys-247 in the C-terminus of 14-3-3ω (see Figure 3a). Here, we confirmed their results and, in addition, showed that at pH 7.5, Mg^{2+} had almost the same effect on the elevation of trypsin cleavage sensitivity at the C-terminal Lys-247 site of 14-3-3ω (Figure 3b). In the presence of Mg^{2+} or Ca^{2+}, complete trypsin cleavage at this site was achieved by 4 h compared with about 50% completion in the cleavage reaction without Me^{2+} (Figure 3b). This was consistent with the notion that the activation of the 14-3-3s is not specific to

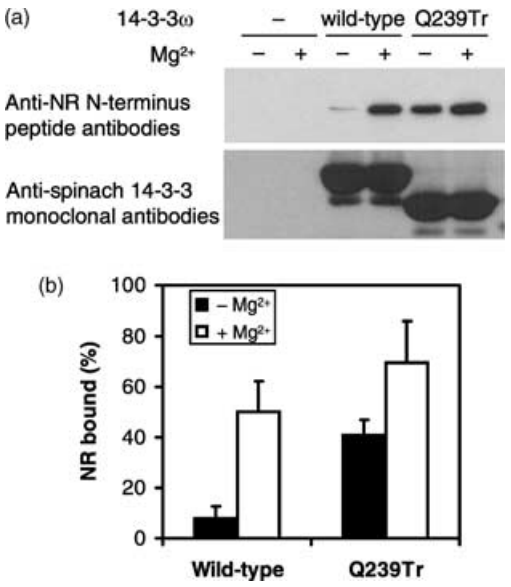

Figure 2. Binding of pNR to immobilized wild-type and the Q239Tr C-terminal truncated form of *Arabidopsis* 14-3-3ω.
(a) Representative immunoblot results showing binding of partially purified spinach pNR in the absence or presence of 5 mM Mg^{2+} to $(His)_6$-tagged 14-3-3 recombinant proteins that were immobilized on the Ni-iminodiacetic acid (Ni-IDA) resin. Resin without bound 14-3-3 proteins was used as a control. Bound proteins were detected by immunoblotting with polyclonal antibodies raised against a spinach NR N-terminal peptide. Monoclonal antibodies to spinach 14-3-3 proteins (Bachmann *et al.*, 1996b) were also used to show that equal amounts of 14-3-3 proteins were bound to the resin.
(b) Densitometry analysis of pNR binding to the wild-type 14-3-3ω or the Q239Tr truncation mutant from three experiments. Bound NR was expressed as a percentage of the NR applied to the column.

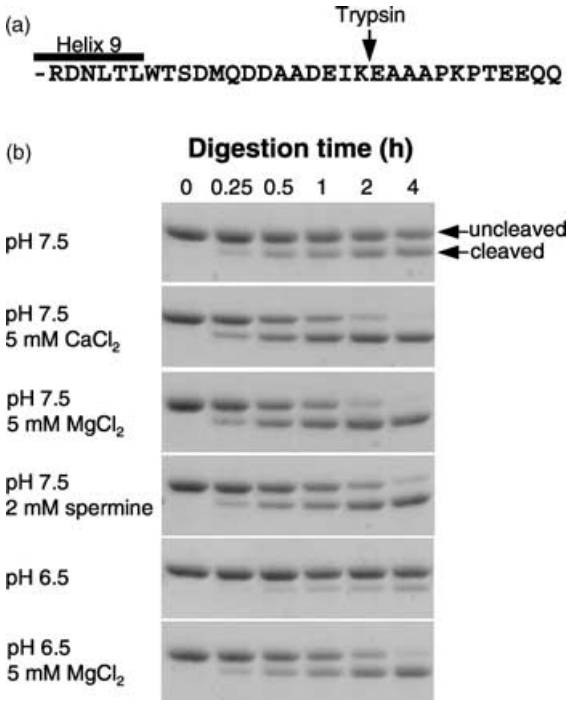

Figure 3. Trypsin cleavage sensitivity of the C-terminus of *Arabidopsis* 14-3-3ω.
(a) The Lys-247 trypsin cleavage site at the C-terminus of 14-3-3ω (Lu *et al.*, 1994) is shown.
(b) Wild-type 14-3-3ω at 200 μg ml^{-1} was incubated with 0.8 μg ml^{-1} of trypsin for up to 4 h in various conditions, as indicated in the panels, and proteins were then separated with SDS–PAGE and stained with Coomassie Blue. Representative results from two independent experiments are shown.

the binding of a particular type of Me^{2+}. Moreover, at pH 7.5, the polyamine spermine at 2 mM also induced trypsin sensitivity of the C-terminal tail of 14-3-3ω (Figure 3b). Therefore, Me^{2+} and polyamines may activate 14-3-3 proteins with the same mechanism, i.e. through binding to the loop 8 region and causing conformational changes in the 14-3-3 protein that facilitates easier access of the target protein to the binding groove (Athwal and Huber, 2002). Such changes may include increased exposure of the C-terminal tail to the medium that results in increased sensitivity to trypsin digestion. We verified that the metal ions were not increasing trypsin proteolytic activity in the control experiments where BSA was substituted for 14-3-3ω as the substrate (data not shown).

In the absence of polycations, decreasing pH to 6.5 did not increase the trypsin sensitivity of the 14-3-3ω C-terminus (Figure 3b). When 5 mM Mg^{2+} was included in the pH 6.5 assay buffer, the trypsin sensitivity was elevated to the same extent as observed at pH 7.5, i.e. complete cleavage at the C-terminal site by 4 h (Figure 3b). Thus, using the trypsin sensitivity at the C-terminal tail of 14-3-3ω as an indicator, we confirmed that the activation of the 14-3-3 proteins by polycations at pH 7.5 and by protons at pH 6.5 were mediated by different mechanisms.

The C-terminal tail of 14-3-3ω is not fully accessible to antibodies

To further understand how the C-terminal tail of 14-3-3ω is structurally organized, polyclonal antibodies were raised to a peptide corresponding to the region from Asp-244 to Thr-255 (Figure 4a). SDS–PAGE of equal amounts of the wild type and T255Tr, and subsequent immunoblotting with the antibodies to the C-terminal peptide demonstrated similar antibody binding to both the antigens in denaturing conditions (Figure 4b). Immunoprecipitation of the wild-type 14-3-3ω or T255Tr truncation showed that the antibody binding to both the antigens was fast and reached saturation within 5 min (data not shown), indicating that the antigen–antibody interactions were of high affinity. Immunodepletion assays with increasing molar ratios of dimeric antibody to dimeric 14-3-3 showed that it took more than a four- or twofold excess of the antibodies relative to the wild-type or T255Tr 14-3-3ω proteins, respectively, to completely precipitate the antigens (Figure 4c). Because the antibodies were affinity-purified with the peptide antigen as the ligand, the results suggested that neither the wild-type nor the

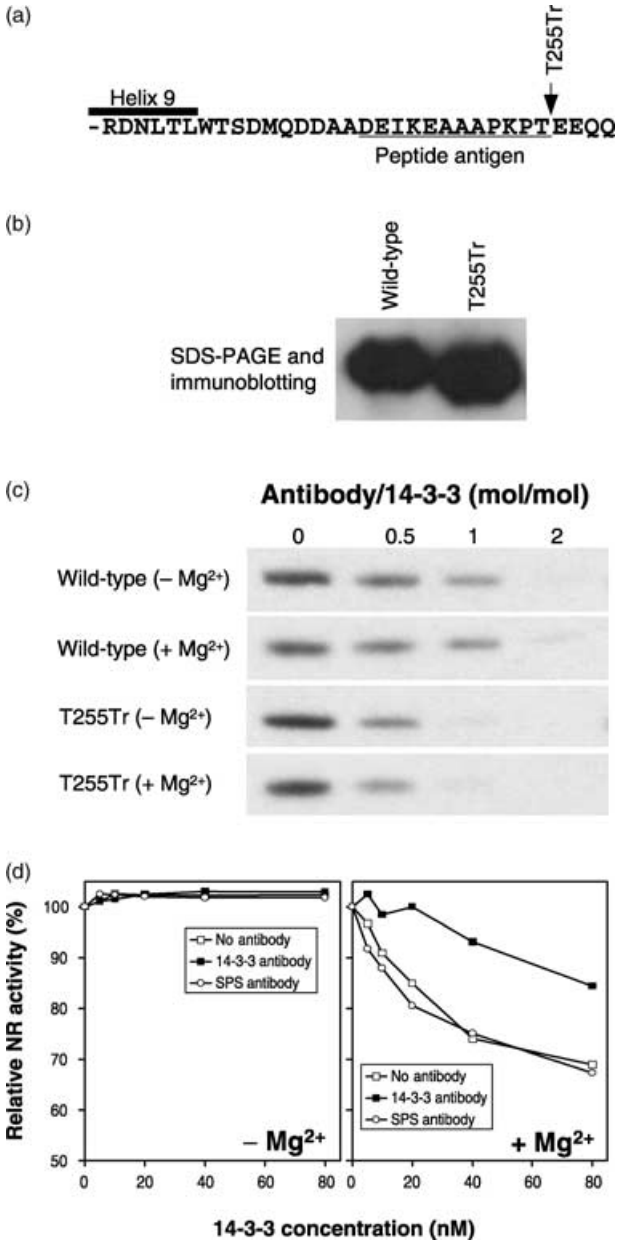

Figure 4. Immunoreactivity of *Arabidopsis* 14-3-3ω to antibodies to its C-terminus.

(a) The C-terminus of 14-3-3ω showing the sequence used to produce polyclonal antibodies.

(b) Binding of the denatured wild-type and the T255Tr truncated form of 14-3-3ω to the C-terminus antibodies is shown by immunoblotting after SDS–PAGE.

(c) Binding of the wild-type or the T255Tr truncation mutant to the polyclonal antibodies in non-denaturing conditions as assayed by immunodepletion. Antibodies and antigens, at the molar ratios indicated, were incubated with protein G-Sepharose either in the absence (–Mg^{2+}) or presence of 5 mM $MgCl_2$ (+Mg^{2+}). Antigen remaining in the supernatant was detected by immunoblotting with monoclonal antibodies to the N-terminus of 14-3-3ω following SDS–PAGE. Reliability of the immunodepletion results was confirmed by at least three independent immunoprecipitation assays, in which precipitated wild-type 14-3-3ω was always approximately 50% of T255Tr (not shown).

(d) Effect of polyclonal antibodies to the 14-3-3ω C-terminal peptide on inhibition of pNR activity by wild-type 14-3-3ω at pH 7.5 either in the absence of $MgCl_2$ (–Mg^{2+}) or in the presence of 5 mM $MgCl_2$ (+Mg^{2+}). The 14-3-3 proteins were incubated with 320 nM of the antibodies at 20°C for 30 min before the pNR inhibition assay. The sucrose-phosphate synthase (SPS) antibodies used as a control were purified rabbit polyclonal antibodies against a synthetic peptide, RGVSCHGRF, corresponding to a central region of spinach SPS.

T255Tr mutant was readily precipitated by the antibodies to the C-terminal peptide, but that the wild type was even slightly less capable of antibody binding than T255Tr. Because the immunodepletion assay was performed in non-denaturing conditions, the 14-3-3 proteins were presumed to have retained their native conformations. Thus, truncation at Thr-255 might have disrupted certain conformations in the C-terminus of the wild-type 14-3-3ω so that the T255Tr protein was bound by the antibodies more readily than the wild type. The C-terminal tail might either assume an ordered secondary structure that diminished certain epitopes in this region and/or was positioned in a way that hindered the access of the antibodies. The lack of any effect of Mg^{2+} on immunoprecipitation of the 14-3-3s (Figure 4c) was surprising, but might be as a result of the rapid and irreversible nature of the antigen–antibody binding in contrast to the slow reaction of the trypsin cleavage, which took at least 4 h to reach completion (Figure 3b). Nevertheless, binding of the wild-type 14-3-3ω to the antibodies could be saturated regardless of the presence of Me^{2+} as shown in the immunodepletion assays. The Mg^{2+}-dependent inhibition of the pNR activity by wild-type 14-3-3ω was reduced in proportion to the amount of 14-3-3 bound to the antibodies (Figure 4d), suggesting that the binding of antibodies to the C-terminus of 14-3-3ω prevented the interaction with pNR.

The C-terminal tail of 14-3-3ω may contain a tenth α-helix

The secondary structure in the C-terminal tail of 14-3-3ω could be responsible for the relatively weak antibody binding under non-denaturing conditions (Figure 4). Because a three-dimensional structure of the C-terminal tail of a 14-3-3 protein has never been experimentally determined, we first carried out *in silico* predictions for the secondary structures of 14-3-3ω with a number of methods (for references, see the Network Protein Sequence Analysis Worldwide Web server at http://npsa-pbil.ibcp.fr/NPSA/npsa_server.html). All these methods predicted that the region will contain an α-helix with a core segment from Asp-240 to Ala-250 (Figure 5a). Many of the other 11 *Arabidopsis* isoforms may also contain an α-helix in their C-terminal tails as predicted by the PHD method (Rost and Sander, 1993; Figure 5b). As the 14-3-3s are known to contain nine α-helices (Liu *et al.*, 1995; Obsil *et al.*, 2001; Xiao *et al.*, 1995), the putative α-helix in the C-terminal tail would constitute a tenth α-helix.

Subsequently, a peptide corresponding to the region from Trp-234 to Lys-253 of 14-3-3ω, encompassing the predicted α-helical structure, was analyzed with circular dichroism (CD; Figure 6a, middle panel). The CD spectrum from 184 to 250 nm did not indicate that the peptide contained any ordered secondary structure in the aqueous solution (Figure 6a, middle panel). However, when trifluoroethanol (TFE), a compound that allows polypeptides to assume secondary structures (Nelson and Kallenbach, 1986), was included and increased to 30 and 45%, the CD spectra demonstrated a tendency to have a minimum at 222 nm, typical of the α-helical structure (Figure 6a, middle panel). In contrast, the CD spectra of a peptide corresponding to the loop 8 region of 14-3-3ω showed that it retained the unordered structure even at 45% TFE (Figure 6a, left panel). Moreover, substituting prolyl residues for Glu-245 and Ile-246 in the C-terminal peptide completely disrupted the helical structure in TFE (Figure 6a, right panel) as expected. The calculated secondary structure of the peptides was computed with the CONTIN/LL method (Provencher and Glöckner, 1981; Sreerama and Woody, 2000) by comparison with a set of 37 proteins of known structures. The C-terminal peptide reached a plateau of 20% α-helical content with 30% TFE, whereas the control loop 8 peptide and the C-terminal peptide with prolyl substitutions assumed very little α-helix even at 45% TFE (Figure 6b). Thus, both *in silico* prediction and CD analysis support the notion that the C-terminal tail of 14-3-3ω, and likely some

(a)

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          Helix 9
          -RDNLTLWTSDMQDDAADEIKEAAAPKPTEEQQ
PHD       hh          hhhhhhhhhhh
GOR1      h        hhhhhhhhhhhhhhhh hhhhhh
GOR3      h   h hhhhhhhhhhhhhhhhh    hhhhh
GOR4      h        hhhhhhhhhhhhh
SOMP      h  h   hhh  hhhhhhhhhhh      hhh
SOMPA     h             hhhhhhhh      hhh
SIMPA96   h           hhhhhhhhhh
HNN       hh  hhhh  hhhhhhhhhhhhh
DPM       h         hh hhhhhhhhhhh    hh
PREDATIR  h           hhhhhhhhh
MLRC      h           hhhhhhhhhh
DSC       h          hhhhhhhhhhh
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(b)

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Nu        -RDNLTLWNSDINDEAGGDEIKEASKHEPEEGKPAETGQ
Upsilon   -RDNLTLWTSDLNDEAGDDIKEAPKEVQKVDEQAQPPPSQ
Psi       -RDNLTLWTSDMTDEAGDEIKEASKPDGAE
Chi       -RDNLTLWASDMQDDVADDIKEAAPAAAKPADEQQS
Phi       -RDNLTLWTSDMQDESPEEIKEAAAPKPAEEQKEI
Omega     -RDNLTLWTSDMQDDAADEIKEAAAPKPTEEQQ
Lambda    -RDNLTLWTSDMQEQMDEA
Kappa     -RDNLTLWTSDMQEQMDEA
Mu        -RDNLTLWTSDISEEGGDDAHKTNGSAKPGAGGDDAE
Iota      -RDNLTLWTSDLPEDGGEDNIKTEESKQEQAKPADATEN
Omicron   -RDNLTLWTSDLEEGGK
Epsilon   -RDNLTLWTSDLNEEGDERTKGADEPQDEN
Zeta      -RDNLTLWTSDTQGDEAEAGEGGEN
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Figure 5. Prediction of α-helical structure in the C-terminus of *Arabidopsis* 14-3-3 isoforms.
(a) Prediction of helical regions for the 14-3-3ω isoform with different methods identified in the left column (for references, see the Network Protein Sequence Analysis Worldwide Web server at http://npsa-pbil.ibcp.fr/NPSA/npsa_server.html). Amino acid residues predicted to assume an α-helical structure are indicated by the letter 'h'. Helix 9 is indicated by the thick bar on top of the sequence.
(b) Helical predictions for the different *Arabidopsis* 14-3-3 isoforms with the PHD method (Rost and Sander, 1993). Amino acid residues predicted to assume an α-helical structure are highlighted and the human 14-3-3ζ isoform is shown as a reference.

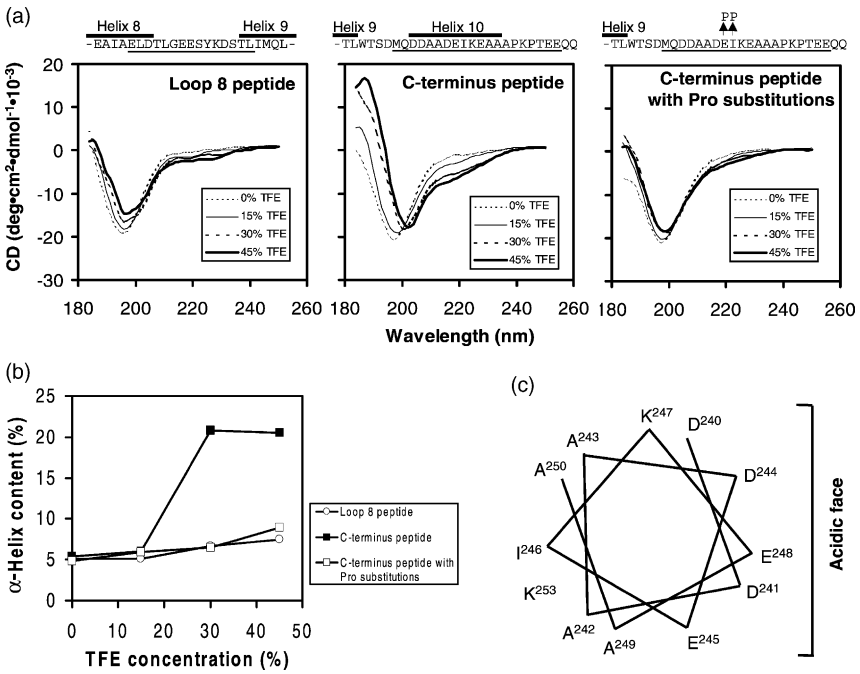

Figure 6. Circular dichroism (CD) analysis of the *Arabidopsis* 14-3-3ω C-terminal peptide.
(a) CD spectra were measured from 184 to 250 nm for a 14-3-3ω loop 8 peptide (left panel), a C-terminal peptide (middle panel), and the C-terminal peptide containing prolyl substitutions for Glu-245 and Ile-246 (right panel), with addition of 0, 15, 30 or 45% (v/v) trifluoroethanol (TFE). Peptide sequences are underlined and predicted helical regions are indicated as thick bars. The curves are averages of mean residue molar ellipticity from four scans of each sample.
(b) Estimation of α-helical contents of the peptides from their CD spectra using the CONTIN/LL method (Provencher and Glöckner, 1981; Sreerama and Woody, 2000) against a set of 37 proteins of known secondary structures.
(c) Helical wheel projection of the putative tenth α-helix in the C-terminal tail of 14-3-3ω.

other plant 14-3-3 isoforms, may contain a tenth α-helix. A helical wheel projection of the putative tenth α-helix suggests that the helix has a distinct acidic face (Figure 6c).

To evaluate the requirement of the tenth α-helix in the C-terminal tail of 14-3-3ω for function in autoinhibition, we made the recombinant 14-3-3ω that contained the double mutant E245P/I246P (see Figure 7a). The double prolyl substitution that was shown to disrupt the helical structure of the corresponding C-terminus synthetic peptide (Figure 6a) had no significant effect on the inhibition of the pNR activity either in the presence or in the absence of Mg^{2+}, at pH 7.5 (Figure 7b). In addition, although the E245P/I246P mutant form of 14-3-3ω showed slight decrease in trypsin sensitivity at Lys-247, both in the presence and absence of Mg^{2+}

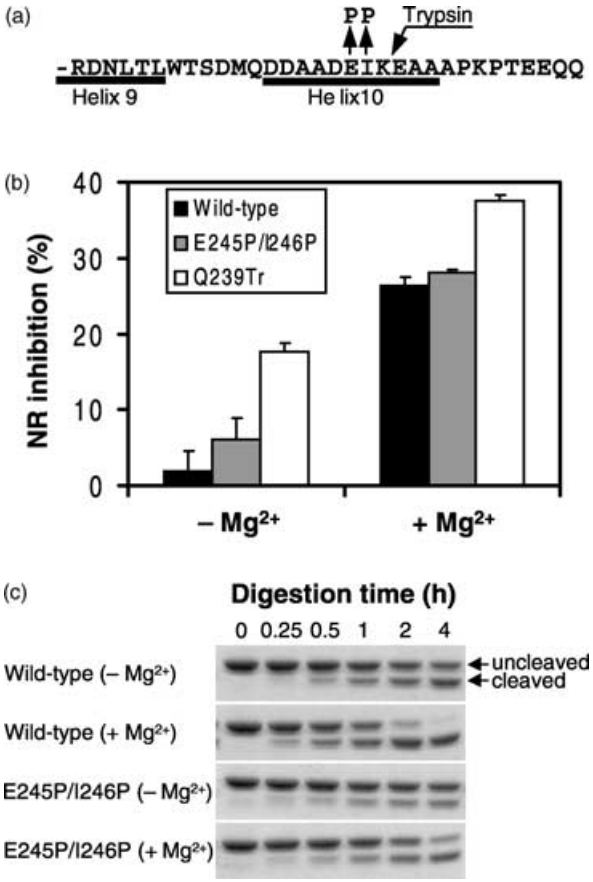

Figure 7. Helix 10 in the *Arabidopsis* 14-3-3ω C-terminus is not required for autoinhibition.
(a) Sequence of the C-terminus of 14-3-3ω showing the E245P/I246P mutation and the trypsin cut site at Lys-247. The double prolyl substitution in the E245P/I246P mutant was constructed to disrupt the putative helix 10, indicated by the thick bar in the C-terminal tail of 14-3-3ω.
(b) Inhibition of pNR activity by the wild-type, Q239Tr truncated form, and E245P/I246P mutant of 14-3-3ω. Assays were done at pH 7.5, either with 80 nM of 14-3-3 proteins in the absence of $MgCl_2$ but with 1 mM EDTA ($–Mg^{2+}$), or with 20 nM of 14-3-3 proteins in the presence of 5 mM $MgCl_2$ ($+Mg^{2+}$). Data are expressed as percentage of inhibition compared with the assay in the absence of 14-3-3 proteins; averages of three determinations are shown and standard errors are indicated.
(c) Trypsin cleavage sensitivity of the E245P/I246P mutant at pH 7.5 either in the absence of $MgCl_2$ ($–Mg^{2+}$) or in the presence of 5 mM $MgCl_2$ ($+Mg^{2+}$) was measured as described in the legend of Figure 3. The trypsin cleavage experiments were repeated once and similar results were obtained.

as compared with the wild-type 14-3-3ω, the Mg^{2+}-induced stimulation of the trypsin cleavage sensitivity was not abolished (Figure 7c). Therefore, the tenth α-helix of 14-3-3ω did not mediate the autoinhibitory function of the C-terminal tail and was not involved in its activation by polycations.

Discussion

Binding of 14-3-3s to pNR is dependent on Mg^{2+}

NADH:nitrate reductase was one of the first plant enzymes shown to be regulated in its activity by the binding of a 14-3-3 protein (Bachmann *et al.*, 1996a; Moorhead *et al.*, 1996). The phosphorylation site on spinach NR has been identified as Ser-543 (Bachmann *et al.*, 1996c; Moorhead *et al.*, 1996), which is located in the hinge 1 region and corresponds to Ser-534 in *Arabidopsis* NR (Su *et al.*, 1996). The inhibition of the pNR activity by 14-3-3 absolutely requires a cation, such as Mg^{2+}, at pH 7.5. The basis for the Mg^{2+} requirement is somewhat controversial, and there are two current models to explain the dependence on Mg^{2+}. One model suggests that the binding of the 14-3-3 to pNR occurs in the absence of Mg^{2+}, but that pNR in the complex is highly sensitive to inhibition by Mg^{2+} (Kaiser *et al.*, 2002). According to the second model, the binding of the 14-3-3 to pNR is promoted by Mg^{2+}, which then forms an inactive pNR:14-3-3 complex. The first model was prompted by the report that the binding of the 14-3-3s to the native NR (as assessed by co-immunoprecipitation) was phosphorylation-dependent, but independent of Mg^{2+} (Weiner and Kaiser, 2000). However, the ability to co-immunoprecipitate pNR with 14-3-3s in the absence of Mg^{2+}, as reported by Weiner and Kaiser (2000), may only be achieved when the enzyme is extracted from the darkened leaves. The NR in the darkened leaves displays hysteretic behavior that had been interpreted as 'tight' binding of a 14-3-3 that is not readily reversed by the removal of Mg^{2+} (Huber and Huber, 1995). This appeared to be the case because we demonstrated in the present study that the binding of partially purified pNR to immobilized 14-3-3ω was strongly stimulated by Mg^{2+} (Figure 2a). It is important to note that the binding of pNR to the 14-3-3ω Q239Tr truncation mutant was substantially independent of Mg^{2+} (Figure 2a), as was inhibition of pNR enzymatic activity (Figure 1b; pH 7.5/–Mg^{2+} plot). Thus, the binding results directly parallel the 14-3-3-dependent inhibition of the pNR activity. We conclude that Mg^{2+} promotes binding of the 14-3-3 to pNR to form an inactive complex. This conclusion is also supported by surface plasmon resonance studies, which demonstrated that binding of the 14-3-3s to an immobilized synthetic phosphopeptide based on the spinach Ser-543 sequence was strongly stimulated by Mg^{2+} (Athwal *et al.*, 2000).

The C-terminal tail in 14-3-3ω is part of the physical basis for autoinhibition

Our results showed that the removal of the C-terminal tail of *Arabidopsis* 14-3-3ω increased its ability to inhibit pNR *in vitro*, as compared to the wild-type 14-3-3ω, at pH 7.5 and 6.5, regardless of the presence of Mg^{2+} (Figure 1). Notably, while the wild-type 14-3-3ω was not able to inhibit pNR at pH 7.5 in the absence of Me^{2+}, the Q239Tr truncated form showed substantial Me^{2+}-independent inhibition of pNR (Figure 1). Binding of the Q239Tr C-terminal truncated form of 14-3-3ω to pNR was also shown to be increased both in the absence and in the presence of Mg^{2+} as compared with the wild-type 14-3-3ω (Figure 2). Thus, we unequivocally demonstrated that the C-terminal tail of a 14-3-3 isoform functions as an autoinhibitor, likely by restricting the interaction with its target protein. We had previously shown that the 14-3-3ω truncation at Trp-234 was unable to inhibit pNR activity in the presence of Mg^{2+} (Athwal and Huber, 2002). These results indicate that the region from Thr-235 to Gln-239 may contain amino acid residues important for 14-3-3 binding, or that extensive truncation causes structural disruption that abolishes 14-3-3 activities. Liu *et al.* (1996) reported that a region in the C-terminus was necessary for the binding of the human 14-3-3τ isoform to a number of its target proteins. Interestingly, the two critical regions in both the 14-3-3 isoforms closely follow helix 9 and overlap for three residues, which are DMQ and DSA in 14-3-3ω and 14-3-3τ, respectively. The Asp residue is conserved in all *Arabidopsis* and human 14-3-3 isoforms except for human 14-3-3β, where there is a conservative substitution of a Glu residue, which is also negatively charged. The role of these residues in maintaining the 14-3-3 protein activity remains to be established.

Liu *et al.* (1996) observed a slight increase in the target protein binding with a truncated form of 14-3-3τ in which two amino acid residues were removed from the C-terminal end, but further truncation either had no additional effect or caused loss of its capability of target protein binding. A recent report indicates that the human 14-3-3ζ C-terminus also behaves as an autoinihibitor for target protein binding (Truong *et al.*, 2002). However, the human 14-3-3 isoforms, with the exception of the ε isoform, have relatively short C-terminal tails compared with most of the plant 14-3-3 proteins as represented by the *Arabidopsis* isoforms (Figure 5b; Yaffe, 2002). The longer, seemingly dispensable C-terminal tails can provide the plant 14-3-3 proteins with certain additional, more sophisticated functions or mechanisms of regulation, for example, differential C-terminal truncation of 14-3-3 isoforms, as observed in the germinated barley embryo (Testerink *et al.*, 2002).

It may be required for the C-terminal tail of 14-3-3ω to be structured orderly to sustain its autoinhibitory activity, for example, a non-structured random coil may not be able to

function as an autoinhibitor and may restrict the binding of pNR to its target protein-binding groove. Both the *in silico* and the CD analyses of a C-terminal peptide indicated that the C-terminal tail had a high propensity to adopt an α-helix structure, which would be the tenth helix in the 14-3-3 protein (Figures 5 and 6). However, the double prolyl substitution in the E245P/I246P mutant that would be expected to disrupt the α-helix, as demonstrated by the CD data from the corresponding peptide with prolyl substitutions (Figure 6), did not significantly alter the pNR inhibition (Figure 7). This suggested that the putative tenth α-helix might not be essential for autoinhibition of 14-3-3ω or for interaction with pNR. However, it remains an open possibility that the tenth α-helix may be necessary for interaction with targets other than pNR or with proteins that regulate the 14-3-3 activity.

Conformational changes in the C-terminus reflect the mechanism of the polycation activation of 14-3-3 proteins

Although the removal of the C-terminal tail of 14-3-3ω makes it partially Me^{2+}-independent for pNR binding and inhibition, the addition of Mg^{2+} further stimulates binding to pNR, and produces greater inhibition of activity (Figures 1 and 2). The Mg^{2+} concentration required for the half-maximal inhibition of pNR by the Q239Tr truncation mutant was essentially the same as that for the wild-type 14-3-3ω. Thus, the C-terminal truncations do not affect the affinity for Mg^{2+} and seem not to physically affect the mechanism of the 14-3-3 activation by polycation binding; only the basal level of pNR inhibition is elevated. The same is true for the activation by protons. However, the observation that trypsin cleavage at Lys-247 in the 14-3-3ω C-terminus was enhanced by the addition of Ca^{2+} (Lu *et al.*, 1994) suggests that conformational changes in the C-terminal tail can reflect the activation of 14-3-3 proteins by Me^{2+}. We showed that Mg^{2+} and spermine, but not low pH, increased the sensitivity of the C-terminus to the trypsin cleavage (Figure 3b). Thus, the C-terminal tail of 14-3-3ω is involved in its activation by polycation binding, but is not totally responsible as the truncated forms of 14-3-3ω still showed some Mg^{2+} effects (Figure 1). The C-terminal trypsin cleavage site at Lys-247 is only partially exposed in the absence of polycations, and polycation binding to the loop 8 region (Athwal and Huber, 2002; Lu *et al.*, 1994) may cause regional structural changes that will include a higher degree of exposure of the cleavage site to the aqueous medium. However, such conformational changes induced by polycations cannot be demonstrated by enhanced binding of 14-3-3ω to the anti-C-terminus antibody (Figure 4c), perhaps because antibody binding is rapid and irreversible.

Taking these data together, we propose the following model for the polycation activation of 14-3-3 proteins. The C-terminal tail of 14-3-3ω is part of a 'gate' that restricts the access of target proteins to the binding groove in the 14-3-3 dimer. Binding of polycations to loop 8 causes conformational changes in the 14-3-3 protein that include, but are not limited to, 'release' of the C-terminus from the internal regions that open the gate so as to facilitate the entrance of the target protein. These conformational changes also include loop 8 because the C-terminal truncated forms of 14-3-3ω still respond to Mg^{2+}. Therefore, displacement of the C-terminus cannot be the only factor resulting from polycation binding. It is likely that the conformational changes in loop 8, or interaction of loop 8 residues with adjacent helices, are also involved.

This model does not apply to the activation of the 14-3-3 proteins by low pH, which is possibly through protonation of certain amino acid residues. The conformational changes of the 14-3-3 protein caused by protonation may be very different from that caused by polycation binding, as the accessibility of the trypsin cleavage site at the C-terminus of 14-3-3ω is not altered at low pH (Figure 3b). Moreover, the effects of polycation binding and protonation seem to be additive to each other and to the C-terminal tail truncation, as demonstrated by the pNR inhibition activity of the Q239Tr truncated form of 14-3-3ω in the presence of Mg^{2+} at pH 6.5 (Figure 1b). Thus, we can conclude that there are three independent mechanisms for 14-3-3 activation observed *in vitro*: (i) polycation binding, (ii) protonation, and (iii) release of the C-terminal tail autoinhibition.

The variant C-terminal tails may determine isoform-specific functions of the 14-3-3 proteins

We have demonstrated that the C-terminal tail of 14-3-3ω functions as an autoinhibitor *in vitro*. The significance of such regulation *in vivo* remains elusive. One apparent possibility is that it offers a mechanism for complete dependence of some 14-3-3 isoforms on cytosolic Me^{2+}, polyamines, or pH. Only in certain cellular conditions, such as an increase of Me^{2+} or polyamine concentration or decrease in cytosolic pH, would the 14-3-3 proteins be active in target protein binding. On the other hand, proteolytic removal of the C-terminal tail *in vivo* could be a mechanism that cells use to maximally activate 14-3-3 proteins. For example, C-terminal truncation was observed during barley seed germination (Testerink *et al.*, 2002; van Zeijl *et al.*, 2000). Although the consequence of this C-terminal truncation of a 14-3-3 protein is not known, proteolytic processing is not an unusual mechanism that cells use to activate proteins.

The sequence divergence of the C-terminus among isoforms implies that this region may mediate interactions with other proteins and may be involved in determining the specificity of interaction between 14-3-3 proteins and their target proteins. It was observed that different *Arabidopsis* 14-3-3 isoforms had different inhibitory activities on pNR (Bachmann *et al.*, 1996a). Each 14-3-3 target protein might

have a particular type of 14-3-3 dimer as its preferred or optimal regulator. Removing the C-terminal tails of other 14-3-3 isoforms and swapping the C-terminal tails between them should give some insight into this question.

Experimental procedures

NADH:nitrate reductase extraction, phosphorylation, and activity assay

Extraction, partial purification, and *in vitro* phosphorylation of spinach (*Spinacia oleracea* L.) leaf NR was as described previously, with slight modifications (Bachmann *et al.*, 1995, 1996b,c; Huber and Huber, 1995). Briefly, 20 g of light-harvested spinach leaves were ground on ice in 40 ml of cold extraction buffer (50 mM 3-(N-morpholino) propanesulfonic acid-NaOH (MOPS–NaOH), pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA, 2.5 mM DTT, and 0.1% (v/v) Triton X-100) for 10 min. After centrifugation, the clarified crude extract was subjected to polyethylene glycol (average molecular weight 8000) precipitation from 3 to 13% (v/v). The precipitated proteins were dissolved in 10 ml of 50 mM MOPS–NaOH, pH 7.5, 10 mM $MgCl_2$, and 2.5 mM DTT, and the solution was passed through a 0.45 μm filter before being applied to a 1 ml column of SOURCE 15Q anion exchanger (Pharmacia, Uppsala, Sweden) for fast protein liquid chromatography in the same buffer. NR was eluted with a 40 ml NaCl concentration gradient from 0 to 500 mM. Fractions of 1 ml were collected and assayed for NR activity, as described below, both in the presence and in the absence of Mg^{2+}. The NR-active fractions were pooled and incubated in 50 mM MOPS–NaOH, pH 7.5, 1 mM ATP, 10 mM $MgCl_2$, 2 mM DTT, and 1 μM microcystin-LR (Calbiochem, La Jolla, CA, USA) at 25°C for 20 min to phosphorylate NR with the coeluting Ca^{2+}-dependent protein kinase (Athwal *et al.*, 1998b) that could be assayed by the phosphorylation activity on a peptide corresponding to the 14-3-3 protein binding site in NR, GPTLKRTASTPFMNTTSK (Bachmann *et al.*, 1996c; McMichael *et al.*, 1995). Phospho-NR was dialyzed against 10 mM MOPS–NaOH, pH 7.5, 2.5 mM DTT, 5 mM EDTA, and 1 mM EGTA, then to the same buffer without EDTA or EGTA, and then stored in 50% (v/v) glycerol at −20°C.

NADH:nitrate reductase activity was measured in a buffer containing 50 mM MOPS–NaOH, pH 7.5, 10 mM KNO_3, and 0.1 mM NADH with either 1 mM EDTA for the '$-Mg^{2+}$' assay or with 5 mM $MgCl_2$ for the '$+Mg^{2+}$' assay (Huber and Huber, 1995). Reactions were incubated for 15 min at 25°C and then stopped by addition of 50 μM zinc acetate. After centrifugation, nitrite in the supernatant was measured as described previously (Huber and Huber, 1995). For assaying pNR inhibition by 14-3-3 proteins, the recombinant 14-3-3 proteins at the concentrations specified in the text, were incubated with pNR for 5 min on ice before NADH was added to initiate the reaction. For assaying Mg^{2+} dependence of 14-3-3 inhibition of pNR, various concentrations of $MgCl_2$ were included in the assay with 30 nM of the dimeric wild-type recombinant 14-3-3ω or with 7.5 nM of its Q239Tr truncated form. Experiments concerning NR activity assay were repeated at least twice to confirm reproducibility, and data from only one typical experiment are shown.

Site-directed mutagenesis and recombinant protein production

The *Escherichia coli* strain overexpressing the $(His)_6$-tagged wild-type *Arabidopsis* 14-3-3ω (Lu *et al.*, 1994), constructed in the vector pET15b (Novagen, Madison, WI, USA), was kindly provided by R.J. Ferl (Department of Horticultural Sciences, University of Florida, Gainesville, FL, USA), and its plasmid was used for site-directed mutagenesis to create the C-terminal truncations and the E245P/I246P mutant forms of 14-3-3ω with the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The complementary primer pairs used for producing nicked double-stranded plasmids were synthesized by Integrated DNA Technologies (Coralville, IA, USA); their sequences are available upon request. The procedures, including thermal cycles with the Pfu DNA polymerase, *Dpn*I digestion, and transformation of the *Escherichia coli* XL-10 Gold strain, followed Stratagene's instructions. The mutants were confirmed with DNA sequencing (service provided by the Duke DNA Analysis Facility, Duke University, Durham, NC, USA), and then the mutated plasmids were introduced to the *E. coli* strain BL21 (DE3).

Recombinant proteins were produced by inducing bacterial cultures grown to log phase with 0.1 mM of isopropyl β-D-thiogalactopyranoside at 37°C for 3 h. Bacteria were pelleted, washed in the binding buffer for $(His)_6$-tag affinity purification (Novagen, Madison, WI, USA), frozen at −20°C overnight, re-suspended in the binding buffer, and lysed by sonication for 3 min. Bacterial debris was removed by centrifugation, and recombinant proteins were purified with 1 ml columns of the His-bind Ni-IDA agarose resin (Novagen) under non-denaturing conditions, according to the manufacturer's instructions. Bound proteins were eluted with the elution buffer (Novagen) and dialyzed against 20 mM Tris–HCl, pH 8.4, 150 mM NaCl, and 2.5 mM $CaCl_2$. The $(His)_6$-tagged proteins were cleaved by biotinylated-thrombin (Novagen) at 20°C for 4 h, with 0.5 unit of enzyme for each milligram of substrate and at a minimal enzyme concentration of 2 units ml^{-1}. Thrombin was removed by streptavidin agarose (Novagen). Cleaved proteins were dialyzed against 10 mM MOPS–NaOH, pH 7.5, 2.5 mM DTT, and 1 mM EDTA, then to the same buffer without EDTA, and stored at 4°C. The resulting recombinant 14-3-3 proteins contained an extra tripeptide sequence of GSH at their N-termini, but the 14-3-3 dimer formation was not affected as revealed by the non-denaturing PAGE (data not shown).

Trypsin digestion

Mild trypsin digestion of 14-3-3ω results only in cleavage of the protein at its C-terminal Lys-247 site as originally demonstrated by Lu *et al.* (1994). Five micrograms of 14-3-3 proteins were mixed with 0.02 μg of bovine pancreas trypsin (Boehringer Mannheim, Germany), with an enzyme to substrate ratio of 1 : 250, in 50 μl of 20 mM MOPS–NaOH, pH 7.5 or 6.5, with addition of 5 mM $CaCl_2$, 5 mM $MgCl_2$, or 2 mM spermine, as indicated in the text. The reactions were carried out at 25°C for up to 4 h and stopped at various times by the addition of the SDS–PAGE sample buffer. The digested proteins were separated by SDS–PAGE with a gel concentration of 12% (w/v) and stained with Coomassie Blue.

Antibody production and immunoassay for 14-3-3 proteins

Polyclonal antibodies were produced in rabbits against a synthetic peptide corresponding to the C-terminus of 14-3-3ω, and affinity-purified by binding to the agarose-coupled antigen peptide (services provided by Bethyl Laboratories, Montgomery, TX, USA). The peptide sequence was CDEIKEAAAPKPT, with an extra Cys residue at the N-terminus to facilitate coupling. The mouse monoclonal antibodies were against an epitope in the N-terminal region of 14-3-3ω and have been described previously (Bachmann *et al.*, 1996b).

For immunoblotting, proteins were separated with SDS–PAGE on 12% gels and electrotransferred to Immobilon-P PVDF

membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 1% (w/v) BSA and 1 M glycine in PBS (5 mM NaH_2PO_4, pH 7.4, 150 mM NaCl) at 20°C for 1 h, and incubated with either the rabbit polyclonal antibodies or mouse monoclonal antibodies in PBS containing 0.1% (v/v) Triton X-100 at 20°C for 1 h. The appropriate horseradish peroxidase-conjugated secondary antibodies were then incubated with the membrane at 20°C for 1 h. After extensive washing in PBS containing Triton X-100, signals were visualized with the ECL reagents (Amersham Pharmacia, Piscataway, NJ, USA), according to the provider's instructions.

For immunodepletion assays, 15 μl packed volume of Protein G-Sepharose (Sigma, St Louis, MO, USA) was incubated with 0, 80, 160, or 320 pmol of the polyclonal antibodies to the 14-3-3ω C-terminal peptide at 20°C for 1 h in 20 mM MOPS–NaOH, pH 7.5, 150 mM NaCl, and 0.1% (v/v) Triton X-100, washed, and incubated again with 160 pmol of the recombinant 14-3-3 protein dimers at 20°C for 20 min in 500 μl of the same buffer, with or without the addition of 5 mM $MgCl_2$. After centrifugation, 20 μl of the supernatant was mixed with an equal volume of double-strength SDS–PAGE sample buffer. Proteins were separated in 12% gels with SDS–PAGE and detected by immunoblotting with the monoclonal antibody to the N-terminus of 14-3-3ω.

Binding of pNR to immobilized 14-3-3 proteins

Affinity purified $(His)_6$-tagged recombinant 14-3-3 proteins were dialyzed against 10 mM MOPS–NaOH, pH 7.5, 2.5 mM DTT, and 1 mM EDTA and then to the same buffer without EDTA. The His-Bind Ni-IDA resin was equilibrated with 50 mM $NiSO_4$ in 0.1% Tween 20 and washed with the pulldown buffer (20 mM MOPS–NaOH, pH 7.5, 100 mM NaCl, 0.1% Tween 20, and 5 mM imidazole). Two hundred and fifty pico moles of dimeric $(His)_6$-tagged 14-3-3 proteins were incubated at 20°C for 30 min with 10 μl bed volume of the His-Bind Ni-IDA resin in 1 ml of the pulldown buffer. The resin was then washed with the pulldown buffer, with or without 5 mM $MgCl_2$ and incubated at 20°C for 20 min with 100 μl of the pNR preparation in 1 ml of the pulldown buffer containing 4 mM NaF and 0.5 μM microcystin-LR, with or without the addition of 5 mM $MgCl_2$. Unbound proteins were removed by washing the resin with the pulldown buffer containing 15 mM imidazole, with or without $MgCl_2$. Finally, the bound proteins were eluted with the SDS–PAGE sample buffer and boiled at 100°C for 5 min. Eluted proteins were separated on 10% gels by SDS–PAGE, and NR was detected by immunoblotting with the rabbit polyclonal antibodies (Bethyl Laboratories, Montgomery, TX, USA) against the peptide, VDRQYHPAPMSGV, corresponding to a region at the N-terminus of the spinach NR. Twenty-five microlitres of the pNR preparation was also loaded for measuring the NR input. Signals on X-ray films were quantified with a densitometer (Molecular Dynamics, Sunnyvale, CA, USA). Bound NR was expressed as a fraction of the NR input. The membranes were also immunodecorated with the monoclonal antibodies to spinach 14-3-3 proteins (Bachmann *et al.*, 1996b) to demonstrate equal amounts of the 14-3-3 proteins bound to the resin.

Circular dichroism analysis and protein secondary structure prediction

The peptides for CD analysis, $ELDTLGEESYKDSTL_{NH2}$ and $WTSDMQDDAADEIKEAAAPK_{NH2}$, corresponding to the loop 8 and the C-terminus regions of 14-3-3ω, respectively, were diluted to 50 μM in 10 mM MOPS–NaOH, pH 7.5, with 0, 15, 30, or 45% (v/v) of TFE. The C-terminus peptide with prolyl substitutions at the corresponding Glu-245 and Ile-246 positions ($WTSDMQDDAA-DPPKEAAAPK_{NH2}$) was also assayed. Ellipticity was measured with a J-600 spectrometer (Jasco, Easton, MD, USA) at 25°C. The optical path of the cuvette was 0.1 cm, and the scans were done from 184 to 250 nm with a bandwidth of 1 nm and step resolution of 0.1 nm. Four scans were run for each sample and the resulting averaged spectra were smoothed. Data are expressed as mean residue molar ellipticities. Estimation of the secondary structure contents was done with the software package available at http://lamar.colostate.edu/~sreeram/CDPro/ (provided by N. Sreerama, Department of Biochemistry and Molecular Biology, Colorado state University, Fort Collins, CO, USA). The CONTIN/LL method (Provencher and Glöckner, 1981; Sreerama and Woody, 2000) was used for the analysis in which a set of 37 proteins with known secondary structures was compared with the experimental results.

A number of methods were used to predict the protein secondary structures according to their amino acid residue sequences, and the prediction was carried out on the Network Protein Sequence Analysis Worldwide Web server at http://npsa-pbil.ibcp.fr/NPSA/npsa_server.html, provided by the Institut de Biologie et Chimie des Protéines, Universite Claude Bernard Lyon1, Lyon, France (references are listed therein).

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References

Athwal, G.S. and Huber, S.C. (2002) Divalent cations and polyamines bind to loop 8 of 14-3-3 proteins, modulating their interaction with phosphorylated nitrate reductase. *Plant J.* **29**, 119–129.

Athwal, G.S., Huber, J.L. and Huber, S.C. (1998a) Biological significance of divalent metal ion binding to 14-3-3 proteins in relationship to nitrate reductase interaction. *Plant Cell Physiol.* **39**, 1065–1072.

Athwal, G.S., Huber, J.L. and Huber, S.C. (1998b) Phosphorylated nitrate reductase and 14-3-3 proteins. Site of interaction, effects of ions, and evidence for an AMP-binding site on 14-3-3 proteins. *Plant Physiol.* **118**, 1041–1048.

Athwal, G.S., Lombardo, C.R., Huber, J.L., Masters, S.C., Fu, H. and Huber, S.C. (2000) Modulation of 14-3-3 protein interactions with target polypeptides by physical and metabolic effectors. *Plant Cell Physiol.* **41**, 523–533.

Bachmann, M., Huber, J.L., Athwal, G.S., Wu, K., Ferl, R.J. and Huber, S.C. (1996a) 14-3-3 proteins associate with the regulatory phosphorylation site of leaf nitrate reductase in an isoform-specific manner and reduce dephosphorylation of Ser-543 by endogenous protein phosphatases. *FEBS Lett.* **398**, 26–30.

Bachmann, M., Huber, J.L., Liao, P.-C., Gage, D.A. and Huber, S.C. (1996b) The inhibitor protein of phosphorylated nitrate reductase from spinach (*Spinacia oleracea*) leaves is a 14-3-3 protein. *FEBS Lett.* **387**, 127–131.

Bachmann, M., McMicheal, R.W. Jr, Huber, J.L., Kaiser, W.M. and Huber, S.C. (1995) Partial purification and characterization of a calcium-dependent protein kinase and an inhibitor protein required for inactivation of spinach leaf nitrate reductase. *Plant Physiol.* **108**, 1083–1091.

Bachmann, M., Shiraishi, R.W., Campbell, W.H., Yoo, B.-C., Harman, A.C. and Huber, S.C. (1996c) Identification of Series-543 as the major regulatory phosphorylation site in spinach leaf nitrate reductase. *Plant Cell*, **8**, 505–517.

Chung, H.-J., Sehnke, P.C. and Ferl, R.J. (1999) The 14-3-3 proteins: cellular regulators of plant metabolism. *Trends Plant Sci.* **4**, 367–371.

Huber, S.C., Bachmann, M. and Huber, J.L. (1996) Post-translational regulation of nitrate reductase activity: a role for Ca^{2+} and 14-3-3 proteins. *Trends Plant Sci.* **1**, 432–438.

Huber, S.C. and Huber, J.L. (1995) Metabolic activators of spinach leaf nitrate reductase: effects on enzymatic activity and dephosphorylation by endogenous phosphatases. *Planta*, **196**, 180–189.

Igarashi, D., Ishida, S., Fukazawa, J. and Takahashi, Y. (2001) 14-3-3 proteins regulate intracellular localization of the bZIP transcriptional activator RSG. *Plant Cell*, **13**, 2483–2497.

Kaiser, W.M. and Huber, S.C. (2001) Post-transcriptional regulation of nitrate reductase: mechanism, physiological relevance and environmental triggers. *J. Exp. Bot.* **52**, 1981–1989.

Kaiser, W.M., Weiner, H., Kandlbinder, A., Tsai, C.-B., Rockel, P., Sonoda, M. and Planchet, E. (2002) Modulation of nitrate reductase: some new insights, an unusual case and a potentially important side reaction. *J. Exp. Bot.* **53**, 875–882.

Korthout, H.A.A.J. and de Boer, A.H. (1994) A fusicoccin binding protein belongs to the family of 14-3-3 brain protein homologs. *Plant Cell*, **6**, 1681–1692.

Liu, D., Bienkowska, J., Petosa, C., Collier, R.J., Fu, H. and Liddington, R. (1995) Crystal structure of the ζ isoform of the 14-3-3 protein. *Nature*, **376**, 191–194.

Liu, Y.-C., Elly, C., Yoshiba, H., Bonnefoy-Berard, N. and Altman, A. (1996) Activation-modulated association of 14-3-3 proteins with Cbl in T-cells. *J. Biol. Chem.* **271**, 14591–14595.

Lu, G., DeLisle, A.J., de Vetten, N.C. and Ferl, R.J. (1992) Brain proteins in plants: an *Arabidopsis* homolog to neurotransmitter pathway activators is part of a DNA-binding complex. *Proc. Natl. Acad. Sci. USA*, **89**, 11490–11494.

Lu, G., Sehnke, P.C. and Ferl, R.J. (1994) Phosphorylation and calcium-binding properties of an *Arabidopsis* GF14 brain protein homolog. *Plant Cell*, **6**, 501–510.

MacKintosh, C. and Meek, S.E.M. (2001) Regulation of plant NR activity by reversible phosphorylation, 14-3-3 protein and proteolysis. *Cell Mol. Life Sci.* **58**, 205–214.

Marra, M., Fullone, M.R., Fogliano, V., Mattei, M., Masi, S. and Aducci, P. (1994) The 30 kilodalton protein present in purified fusicoccin receptor preparations is a 14-3-3-like protein. *Plant Physiol.* **106**, 1497–1501.

McMichael, R.W., Bachmann, M. and Huber, S.C. (1995) Spinach leaf sucrose-phosphate synthase and nitrate reductase are phosphorylated/inactivated by multiple protein kinases *in vitro*. *Plant Physiol.* **108**, 1077–1082.

Moorhead, G., Douglas, P., Cotelle, V. *et al.* (1999) Phosphorylation-dependent interactions between enzymes of plant metabolism and 14-3-3 proteins. *Plant J.* **18**, 1–12.

Moorhead, G., Douglas, P., Morrice, N., Scarabel, B., Aitken, A. and MacKintosh, C. (1996) Phosphorylated nitrate reductase from spinach leaves is inhibited by 14-3-3 proteins and activated by fusicoccin. *Curr. Biol.* **6**, 1104–1113.

Nelson, J.W. and Kallenbach, N.R. (1986) Stabilization of the ribonuclease S-peptide α-helix by trifluoroethanol. *Proteins Struct. Funct. Genet.* **1**, 211–217.

Obsil, T., Ghirlando, R., Klein, D.C., Ganguly, S. and Dyda, F. (2001) Crystal structure of the 14-3-3ζ:serotonin N-acetyltransferase complex: a role for scaffolding in enzyme regulation. *Cell*, **105**, 257–267.

Oecking, C., Eckerskorn, C. and Weiler, E.W. (1994) The fusicoccin receptor of plants is a member of the 14-3-3 superfamily of eukaryotic regulatory proteins. *FEBS Lett.* **352**, 163–166.

Pan, S., Sehnke, P.C., Ferl, R.J. and Gurley, W.B. (1999) Specific interactions with TBP and TFIIB *in vitro* suggest that 14-3-3 proteins may participate in the regulation of transcription when part of a DNA binding complex. *Plant Cell*, **11**, 1591–1602.

Provencher, S.W. and Glöckner, J. (1981) Estimation of protein secondary structure from circular dichroism. *Biochemistry*, **20**, 33–37.

Roberts, M.R. (2000) Regulatory 14-3-3 protein–protein interactions in plant cells. *Curr. Opin. Plant Biol.* **3**, 400–405.

Rosenquist, M., Alsterfjord, M., Larsson, C. and Sommarin, M. (2001) Data mining the *Arabidopsis* genome reveals fifteen 14-3-3 genes. Expression is demonstrated for two out of five novel genes. *Plant Physiol.* **127**, 142–149.

Rosenquist, M., Sehnke, P., Ferl, R.J., Sommarin, M. and Larsson, C. (2000) Evolution of the 14-3-3 protein family: does the large number of isoforms in multicellular organisms reflect functional specificity? *J. Mol. Evol.* **51**, 446–458.

Rost, B. and Sander, C. (1993) Prediction of protein secondary structure at better than 70% accuracy. *J. Mol. Biol.* **232**, 584–599.

Schultz, T.F., Medina, J., Hill, A. and Quatrano, R.S. (1998) 14-3-3 proteins are part of an abscisic acid–VIVIPAROUS1 (VP1)-response complex in the *Em* promoter and interact with VP1 and EmBP1. *Plant Cell*, **10**, 837–847.

Sehnke, P.C., DeLille, J.M. and Ferl, R.J. (2002) Consummating signal transduction: the role of 14-3-3 proteins in the completion of signal-induced transitions in protein activity. *Plant Cell*, **14**, S339–S354.

Sreerama, N. and Woody, R.W. (2000) Estimation of protein secondary structure from CD spectra: comparison of CONTIN, SELCON and CDSSTR methods with an expanded reference set. *Anal. Biochem.* **287**, 252–260.

Su, W., Huber, S. and Crawford, N.M. (1996) Identification *in vitro* of a post-translational regulatory site in the hinge 1 region of *Arabidopsis* nitrate reductase. *Plant Cell*, **8**, 519–527.

Testerink, C., van Zeijl, M.J., Drumm, K., Palmgren, M.G., Collinge, D.B., Kijne, J.W. and Wang, M. (2002) Post-translational modification of barley 14-3-3A is isoform-specific and involves removal of the hypervariable C-terminus. *Plant Mol. Biol.* **50**, 535–542.

Toroser, D., Athwal, G.S. and Huber, S.C. (1998) Site-specific regulatory interaction between spinach leaf sucrose-phosphate synthase and 14-3-3 proteins. *FEBS Lett.* **435**, 110–114.

Truong, A.B., Masters, S.C., Yang, H. and Fu, H. (2002) Role of the 14-3-3 C-terminal loop in ligand interaction. *Proteins*, **49**, 321–325.

Tzivion, G. and Avruch, J. (2002) 14-3-3 proteins: active cofactors in cellular regulation by serine/threonine phosphorylation. *J. Biol. Chem.* **277**, 3061–3064.

Weiner, H. and Kaiser, W.M. (2000) Binding of 14-3-3 proteins is not sufficient to inhibit nitrate reductase in spinach leaves. *FEBS Lett.* **480**, 217–220.

Xiao, B., Smerdon, S.J., Jones, D.H., Dodson, S.G., Soneji, Y., Aitken, A. and Gamblin, S.J. (1995) Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. *Nature*, **376**, 188–191.

Yaffe, M.B. (2002) How the 14-3-3 proteins work? Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett.* **513**, 53–57.

van Zeijl, M.J., Testerink, C., Kijne, J.W. and Wang, M. (2000) Subcellular differences in post-translational modification of barley 14-3-3 proteins. *FEBS Lett.* **473**, 292–296.